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Impacts of ionising radiation on sperm quality, DNA integrity and post-fertilisation development in marine and freshwater crustaceans

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ABSTRACT

Crustaceans have been designated as internationally important model organisms in the development of environmental radioprotection measures. Despite the known sensitivity of sperm to ionizing radiation, the impacts of chronic radiation exposure on male fertility in crustaceans have not been studied. For the first time, the present study aimed to assess the impacts of chronic radiation exposure on male fertility, sperm DNA damage and concomitant impacts on breeding in two amphipod crustaceans. *Echinogammarus marinus* and *Gammarus pulex* (male fertility only) were exposed to phosphorus-32 at dose rates of 0, 0.1, 1 and 10 mGy/d and sperm parameters, DNA damage and knock-on impacts on breeding were assessed. Sperm quality parameters and DNA damage were assessed using a fluorescent staining method and single cell gel electrophoresis respectively. Concomitant effects of male exposure to radiation on fecundity were determined by pairing phosphorus-32 exposed males to unexposed sexually mature females. In *E. marinus,* a statistically significant reduction of 9 and 11% in the quality of sperm was recorded at dose rates of 1 and 10 mGy/d respectively, with no significant effects recorded on sperm counts. Conversely in the freshwater *G. pulex*, no significant impact of radiation on sperm quantity or quality was recorded. For *E. marinus*, a statistically significant increase in DNA damage was recorded at doses of 10 mGy/d. Reduced fecundity and an increase in the frequency of abnormal embryos was recorded in female *E. marinus* breeding with males exposed to radiation. These findings suggest sperm quality may be a sensitive indicator of radiation exposure in invertebrates with potential impacts on the unexposed embryo, though unclear dose-response and differences between two closely related species necessitate further study before robust conclusions can be drawn.

1. Introduction

Radioactive substances are discharged in to the environment both as part of the normal operation of nuclear facilities and accidental releases such as those at the Chernobyl and Fukushima Dai-ichi nuclear power plants (NPPs). Coupled with the renewal of interest in nuclear power as a low-carbon energy source (Ming et al., 2016) and heightened public concern regarding radiation safety (Orita et al., 2015), protection of the wider environment from radioactive substances has become an issue of scientific and regulatory concern (Bréchignac et al., 2016; ICRP, 2007). However, a consensus on appropriate dose levels for protection of non-human organisms is yet to be agreed owing to a lack of data for many groups of organisms following environmentally relevant exposures (Garnier-Laplace et al., 2015).

Sperm cells are thought to be sensitive to ionising radiation (IR) exposure due to a lack of antioxidant enzymes and DNA repair comparable with other biological systems (Fischbein et al., 1997; Lewis and Ford, 2012; Marques et al., 2014). Studies have demonstrated the sensitivity of sperm to radiation in a range of organisms including birds (Møller et al., 2008), fish (Knowles, 1999) and rodents (Liu et al., 2006). For example, Møller et al. (2008), demonstrated perturbations to sperm behaviour and morphology in barn swallows inhabiting areas of Chernobyl with mean dose rates of 3.74 μGy/hr, which is lower than environmental dose rates in natural high-background areas (e.g. Kerala, India, maximum dose rates of 7.99 μGy/hr (Nair et al., 1999)). Recent studies have focused on the impacts of Fukushima-derived radiation on male fertility in rodents (Okano et al., 2016) and bulls (Yamashiro et al., 2013), finding no significant effect of radiation exposure (maximum mean ambient dose rates of 13.9 μSv/hr for rodents and mean total dose of 8 mGy for bulls) on sperm morphology and

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spermatogenesis respectively. However, no study has considered radiation-induced impacts on male fertility in aquatic invertebrates. Typical endpoints in invertebrate radioecology are biased towards female reproductive success such as egg production, time of hatching and egg mass (Fuller et al., 2015).

IR induces damage to DNA both through direction ionisation and by generation of hydroxyl radicals that attack DNA (Hada and Georgakilas, 2008). DNA damage in sperm cells is of particular importance owing to the potential for transmission across generations and effects on progeny (Aitken and De Iuliis, 2007). This persistence of DNA damage is owing to down-regulation of DNA repair and loss of apoptotic capabilities during spermatogenesis (Lewis and Aitken, 2005; Marchetti and Wyrobek, 2008). A number of studies have demonstrated transgenerational effects of genotoxin exposure on aquatic organisms, typically using high concentrations of model chemical genotoxicants such as methyl methanesulfonate (MMS, e.g. (Lewis and Galloway, 2009)). Though the effects of IR on DNA damage in somatic cells have been well documented in aquatic invertebrates (AlAmri et al., 2012; Han et al., 2014; Parisot et al., 2015) to the authors' knowledge no study has considered effects of IR on sperm and potential transgenerational effects.

Consequently, the present study aimed to assess the impacts of chronic radiation exposure on male fertility, sperm DNA damage and resultant reproductive success in the intertidal amphipod crustacean, *Echinogammarus marinus* and the freshwater amphipod, *Gammarus pulex*. Due to high mortality in control cultures of *G. pulex* during long-term breeding experiments, sperm quality and quantity only was assessed. Crustaceans are one of the ICRPs designated reference animals and plants (RAPs), meaning data derived from these organisms will be used to support the evolving system for environmental radioprotection (Larsson et al., 2015). *E. marinus* is a widespread intertidal amphipod species with a distribution from Norway to Southern Portugal (Maranhão et al., 2001; Alexander et al., 2013). *E. marinus* has gained prominence as a model organism in a range of disciplines including ecology (Maranhão et al., 2001) reproductive biology (Ford et al., 2003; Maranhão and Marques, 2003) and ecotoxicology (Bossus et al., 2014; Yang et al., 2008). *Gammarus pulex* is an important species in freshwater systems, being the most abundant native amphipod species in many areas of Western Europe (Maazouzi et al., 2011). *G. pulex* plays a key ecosystem role in leaf litter degradation and thereby nutrient cycling (Maltby et al., 2002). Owing to its ecological importance, *G. pulex* has been used extensively in ecotoxicological research with an emphasis on feeding assays and measures of leaf shredding efficacy (Maltby et al., 2002; Åbjörnsson et al., 2000).

The specific objectives of this study were as follows: i) to assess the impacts of chronic low-dose radiation (dose rates of 0, 0.1, 1 and 10 mGy/d) exposure on crustacean sperm quality and DNA damage (ii) to determine knock-on impacts of radiation-induced perturbations to sperm on reproductive success and post-fertilization development. Owing to the known detrimental impact of IR on male fertility and previous studies demonstrating pollutant impacts on sperm in amphipods (e.g. Yang et al. (2008),), it was hypothesised that:

a) Ionising radiation would cause a reduction in both the number and quality of sperm produced by both *E. marinus* and *G. pulex*.
b) Perturbations to sperm would be linked with an increase in DNA damage, and concomitant effects on female reproduction such as a reduction in fecundity and increased developmental abnormalities in progeny.

In order to achieve these aims, three separate exposures of male *E. marinus* were conducted and effects on male fertility (sperm quantity and quality), DNA damage and resultant fecundity were monitored. Analysis of male fertility in *G. pulex* was conducted following a single radiation exposure. Moult frequency was monitored as an additional endpoint of radiation exposure.

2. Materials & methods

2.1. Collection and culturing of Echinogammarus marinus & Gammarus pulex

E. marinus were collected from Langstone Harbour, England, UK (50°47′23.13 N, 1°02′37.25 W) at low tide periodically from September 2015 to October 2016. Collected *E. marinus* were housed in aquaria containing aerated seawater at 10 °C with a photoperiod of 12 h light 12 h dark. Individuals were fed *ad libitum* on mixed brown fucoid seaweed. Prior to experimentation, animals were sexed by analysis of the uropods following the description of Sexton & Spooner (Sexton and Spooner, 1940) and visually inspected for parasitic (trematode) infection. Adult males were then acclimated to aerated artificial seawater, AFSW, (33 ppt) for at least two weeks separated from females prior to experimentation.

G. pulex were collected using a 500 µm mesh hand net from the River Ems, England, UK, (N50°51′34.8″,W0°55′45.8″) periodically from April 2015 to February 2016. Upon return to the laboratory, individuals were sorted out from other macroinvertebrates and maintained in river water collected from source at time of sampling. *G. pulex* cultures were kept at 15 °C with a 12 h light dark cycle. Prior to experimentation, individuals were sexed by analysis of the posterior gnathopods (Hume et al., 2005) and visually inspected for presence of acanthocephalan parasites. Adult males with no signs of infection were kept in aerated mineral water (Artificial Freshwater, AFW) (Volvic, France) and fed Elm leaves (*Ulmus x hollandica* 'Vegeta') collected from Southsea Common (50°46′47.8″N, 1°05′15.4″W) for two weeks prior to experimentation. Leaves were pre-conditioned according to the methods of Bloor (2010) whereby organic detritus from the sampling site is mixed with leaves for at least ten days to allow for fungal colonisation.

2.2. Exposure conditions & calculation of dose rates

The beta emitter phosphorus-32 (^{32}P) was used as the exposure source in the present study. Phosphorus-32 is used as a proxy for the beta/gamma doses that are primarily received by human and non-human organisms following nuclear incidents (Petersen, 1965; Priyadarshi et al., 2011; Straume et al., 2003). In addition, ^{32}P is released to the environment from medical and research establishments owing to its uses in radiotherapy and as a tracer in genetic studies (Smith et al., 2011), meaning organisms may be exposed to ^{32}P in the natural environment.

A preliminary experiment (Fuller (2018),) was conducted to elucidate the uptake of ^{32}P in *E. marinus* and *G. pulex* and to derive a concentration ratio (CR) value. Based on this preliminary uptake data and calculations using the ERICA tool (v 1.2), organisms were exposed to a solution of ATP γ-^{32}P (PerkinElmer, UK) and AFSW/AFW at a nominal activity concentration of 62.9 Bq/ml and 14.8 Bq/ml for *E. marinus* and *G. pulex* respectively to achieve a 10 mGy/d dose rate. Dilutions of 10 and 100 fold were performed for 1 and 0.1 mGy/d solutions respectively. AFSW and AFW only were used for control treatments for *E. marinus* and *G. pulex* respectively. Activity concentrations of all exposure solutions were measured in triplicate using a HIDEX 300SL liquid scintillation counter (HIDEX, Finland) and associated MikroWin 2000 software (Version 4.43). Structurally analogous non-radioactive phosphorus (ATP, New England Biolabs, UK) of the same molar concentration was added to all treatments excluding the highest dose rate to ensure total phosphorus remained constant amongst all treatments.

For all exposures, individuals were housed in 50 ml glass beakers (Fisher Brand) containing 20 ml of exposure solution of either control, 0.1, 1 or 10 mGy/d. For each experiment, 24 animals per treatment were exposed for a two week period. *E. marinus* individuals were kept at 10 °C within a cooled incubator (Panasonic MIR-254) with a 12 h light 12 h dark cycle. *E. marinus* individuals were fed on 100 mg pieces (± 10%) of brown fucoid seaweed collected from source. *G. pulex* were

kept at 15 °C under the same photoperiod and fed on 50 mg (± 10%) pre-conditioned elm leaves. In both species, water changes of 18 of the 20 ml of exposure solution were conducted every three days. Activity concentrations in *E. marinus* and *G. pulex* individuals and corresponding exposure medium were analysed using liquid scintillation counting at each water change and individuals were monitored daily for mortality/moults. For *E. marinus* experiments, three exposures were conducted for male fertility, DNA damage and breeding assays respectively. Prior to sperm DNA damage analyses in *E. marinus*, male fertility was also assessed serving as an experimental repeat for the first exposure. In *G. pulex* a single exposure was conducted, and sperm quantity and quality assessed.

2.3. Sperm quality analysis

Following radiation exposure, male *E. marinus* and *G. pulex* individuals were anaesthetised using a clove oil solution described in Venarsky & Wilhelm (Venarsky and Wilhelm, 2006). Testes were dissected following Ford et al. (2005), and placed in 20 µl of a pre-made Leibovitz L-15 Medium (Sigma-Aldrich, UK) and 10 mM HEPES (Fisher Scientific, UK) solution. Testes were dilacerated and the resultant solution was transferred by pipette to a pre-weighed microcentrifuge tube containing 20 µl of L-15/HEPES solution. After mixing by pipette and vortexing for 10 s, the solution was reweighed to obtain the exact volume of liquid and kept on ice. Sperm viability was assessed using a LIVE/DEAD viability kit (Molecular Probes Inc). 5 µl of 10 µM SYBR-14® in dimethyl sulfoxide (Sigma-Aldrich, UK) was added to the sperm solution and vortexed for 10 s. Following dark incubation at room temperature for 5 min, 5 µl of 2.4 mM propidium iodide was added and incubated in darkness for a further 5 min 5 µl aliquots of stained sperm solution were added to the upper and lower grids of a Neubauer bright-line haemocytometer and a cover slip added. Slides were visualised using a Leica DM2000 fluorescent microscope with filters of 340–480 nm and 450–490 nm for SYBR-14 and PI staining respectively. Numbers of live and dead sperm were counted in three randomly selected grid squares of the haemocytometer and three replicate counts performed. Sperm viability (% of live sperm) and total sperm count in the solution was calculated. Analysis of male fertility was conducted on a total of 148 male *E. marinus* over two separate exposures. For *G, pulex*, 61 male *E. marinus* were analysed following a single exposure.

2.4. DNA damage analyses

The alkaline comet assay was used to assess sperm DNA damage in the present study following a method adapted from Lacaze et al. (2011a). Following viability analysis, sperm solutions were mixed with 1% low melting point agarose preheated to 37 °C (Sigma-Aldrich, UK) at a ratio of 50:50 and 30 µl spread on a 20-Well CometSlide HT® (TREVIGEN, USA). This process was repeated for 20 sperm solutions per slide randomly between treatments. Slides were incubated in darkness at 4 °C for 7 min and then submerged in lysis solution (TREVIGEN, USA) for a further 18 h under those conditions. Following lysis, slides were washed twice for 5 min at room temperature with distilled water. Slides were immediately placed in an electrophoresis tank (GeneFlow, UK) containing approximately 600 ml of freshly prepared alkaline electrophoresis solution (300 mmol/L NaOH, 1mmol/L EDTA, pH 13). Electrophoresis was performed at 24 V, 405 mAmp for 15 min. Slides were washed twice for 5 min with neutralisation buffer (Tris-Hcl, 400mmol/L, pH 7.5), followed by one 5 min wash with 70% ethanol. Slides were then archived for later staining and comet scoring. All the steps described above were performed either in darkness or under yellow light to prevent induction of DNA damage from UV light.

Slides were stained with 1 x SYBR Gold (Molecular Probes Inc) and allowed to air dry in darkness. Slides were visualised using a Leica DM2000 high-powered fluorescence microscope and five random photographs taken per spot under 100× magnification using a LEICA DFC130 camera. Comet images were randomly coded by a colleague to prevent bias and analysed using the OpenComet v1.3 software (Gyori et al., 2014). Percentage of tail DNA was used as a measure of DNA damage in the present study (Kumaravel and Jha, 2006).

2.5. Breeding experiment

To ascertain the concomitant impacts of potential perturbations to sperm quality/quantity, radiation exposed male *E. marinus* were allowed to reproduce with a non-exposed female. Following radiation exposure, a total of 81 male *E.marinus* were paired with a sexually mature female. Sexually mature females were defined as those in amplexus, a mating behaviour wherein the male guards the female prior to copulation (Watts et al., 2002). Prior to the experiment, mature females were separated from pairs in culture following the method of Malbouisson et al. (1995). Exposed males were first weighed and added sequentially to individual aquaria containing mature females. Fucoid seaweed was added for feed and the time of male addition recorded. Observations of pairing status (paired or unpaired), moulting and mortality were observed daily. Water changes with fresh AFSW were conducted every three days and feed replenished. Once females had moulted and were ovigerous (bearing eggs), males were immediately removed. After five days post-reproduction, ovigerous females were reweighed, anaesthetised and embryos were removed from the marsupium using a plastic Pasteur pipette and forceps. Embryos were photographed using a Leica MZ10F dissecting microscope with mounted Leica DFC130 camera. Photographs were randomly coded by a colleague to ensure all subsequent analysis was conducted blind. The number of eggs per individual and % embryo abnormalities per female were calculated following Sundelin & Eriksson (Sundelin and Eriksson, 1998) and Sundelin et al. (2008). Abnormalities were scored as abnormal or normal, with no distinction of the severity of the aberration. Embryo diameters were measured using Image J (v1.48).

2.6. Statistical analyses

Pooled moult data for all experiments was analysed using a generalized linear model (GZLM) with the R package lme4 to account for the binomial nature of the moult dataset (Bates et al., 2014). An analysis of covariance (ANCOVA) design was employed to test for differences in sperm numbers in relation to dose rate, with male weight used as a covariate. For sperm quality, a GLM was used with no covariate. For the sperm quality and quantity models in *E. marinus*, exposure (trial 1 or 2) was added as an additional random factor to test for differences in sperm parameters between the two experiments. Differences in the amount of DNA damage between dose rates was assessed using ANOVA on log-transformed % tail DNA data with the R function aov (R Core Team, 2016). Assumptions of the analyses were tested by inspection of the residuals and normality testing using a Shapiro-Wilk test. For the breeding experiment, a Kruskal-Wallis H test was used to test for differences in time taken to reproduce between treatments owing to a non-normal distribution of the dataset. Differences in fecundity between females breeding with IR-exposed males were tested using an ANCOVA design with female weight as a covariate. Differences in embryo diameters and percentage of abnormalities per female were tested using a Kruskal-Wallis H test and a 2×4 contingency table with a subsequent χ^2test respectively. The relationship between observed effects on sperm and resultant female reproduction was assessed using Spearman's rank-order correlation.

3. Results & discussion

3.1. Activity concentrations of exposure medium and dose rates

Mean measured activity concentrations of exposure medium across all three experiments in *E. marinus* (male fertility, DNA damage and

breeding experiment) were 63.6 ± 5.24, 6.31 ± 0.685 and 0.676 ± 0.134 for 10 mGy/d, 1 mGy/d and 0.1 mGy/d treatments respectively. Values were close to the desired concentrations of 62.9, 6.29 and 0.629 Bq/ml required to achieve target dose rates based on a preliminary study of ^{32}P uptake in *Echinogammarus marinus* (data not shown). For *G. pulex,* mean measured activity concentrations over the experiment were 14.623 ± 0.564, 1.455 ± 0.268, 0.270 ± 0.144 for 10 mGy/d, 1 mGy/d and 0.1 mGy/d treatments respectively, close to the target concentrations of 14.8, 1.48 and 0.148.*3.2 Moulting & Mortality*.

Radiation dose rate did not affect the frequency of moulting in *E. marinus* (GLZM, df = 3, χ^2 = 0.467, p > 0.05) or *G. pulex* (GLZM, df = 3, χ^2 = 0.931, p > 0.05). In *E. marinus,* both time (df = 13, χ^2 = 12.441, p < 0.05) and experiment (df = 2, χ^2) = 5.351, p < 0.05) had significant effects on moulting, suggesting that the frequency of moulting events varied both during and between individual radiation exposures. However, the non-significant interaction term between dose rate and time (χ^2 = 0.393, df = 17, p > 0.05) and dose rate and experiment (χ^2) = 1.569, df = 4, p > 0.05) implied that these differences were unrelated to radiation treatments. Mortality across the three exposures in *E. marinus* ranged from 0% in controls to 4.1–25% in 10 mGy/d treatments. Mortality in *G. pulex* exposures was higher, ranging from an average of 14.6 ± 2.9% (mean ± standard deviation) in controls to 25 ± 5.9% in 10 mGy/d treated individuals. The finding of no significant effect of radiation exposure on moulting in *E. marinus* and *G. pulex* is consistent with the work of Hoppenheit (1973) and Hoppenheit and Woodhead (1980), who recorded no significant effect of high doses of X-rays and Americium-241 (^{241}Am) on moulting in the brackish water amphipod, *Gammarus duebeni*.

3.2. Sperm quantity & quality

Radiation dose rate did not affect the numbers of sperm produced by *E. marinus* (see Fig. 1, $F_{1, 136}$ = 1.423, p > 0.05). Mean sperm numbers over the two exposures were 17,919 ± 100 or 11.57 ± 3.17 following fourth root transformation (N = 148). Significant differences in sperm numbers were observed between the two trials (ANCOVA, $F_{1, 136}$ = 193, p < 0.05) with 9.25 ± 1.59 and 13.95 ± 2.56 recorded in trials 1 and 2 respectively. However, a non-significant interaction term between radiation dose rate and trial ($F_{3, 136}$ = 1.173, p > 0.05) suggested the effect of radiation was consistent between experiments. Wet weight had a highly significant effect on sperm numbers ($F_{1, 136}$ = 40.60, p < 0.001).

E. marinus sperm viability (% Live Sperm) decreased in a nonlinear manner which declined monotonically with increasing radiation dose rate (see Fig. 1) with significant reductions of 9 and 11% relative to controls at 1 and 10 mGy/d (ANOVA, Pairwise comparisons, Bonferroni correction, p < 0.01). No significant differences were observed between control and 0.1 mGy/d treated groups (p > 0.05). No significant differences in sperm viability was recorded between trials (ANOVA, $F_{1, 140}$ = 0.707, p > 0.05). Mean sperm viability for the two trials was 86.59 ± 9.20%. Wet weight was not significantly correlated with sperm viability (Regression analysis, $F_{1, 145}$ = 3.334, R^2 = 0.022, p > 0.05).

In *Gammarus pulex*, no significant effect of radiation dose rate on sperm numbers was recorded (ANCOVA, $F_{3, 60}$ = 1.214, p > 0.05). Mean sperm numbers were 3331 ± 2071, or 7.60 ± 6.75 following fourth root transformation (N = 61). Highest sperm numbers were recorded in the control group (8.90 ± 2.65), with individuals in the 1 mGy/d displaying the fewest sperm (5.95 ± 2.17). Wet weight had a highly significant effect on numbers of sperm ($F_{1, 60}$ = 27.39, p < 0.001). For sperm viability, a weak but significant positive relationship between wet weight and arcsine viability was recorded (Regression analysis, $F_{1, 59}$ = 4.042, R^2 = 0.064, p < 0.05) therefore an ANCOVA model was used for subsequent analysis with wet weight as a covariate. Sperm viability was reduced at all dose rates relative to controls, with the lowest sperm viability observed in the 1 mGy/d treatment (69.10 ± 20.47%). However, the effects of radiation on sperm viability in *G. pulex* were non-significant (ANCOVA, $F_{3, 60}$ = 0.883, p > 0.05) owing to high inter-individual variability (see Fig. 1). Mean sperm viability in *G. pulex* across all treatments was 73.55 ± 16.07%.

This study is the first to consider radiation effects on male fertility in aquatic invertebrates. It was hypothesised that radiation would cause a significant reduction in both sperm quantity and quality in *E. marinus* and *G. pulex*. In *E. marinus*, a significant reduction in sperm quality at 1 and 10 mGy/d was recorded, though sperm numbers were unaffected. Conversely for the freshwater amphipod *G. pulex*, no significant effect of radiation on either sperm quality or quantity was recorded. Support for the hypothesis was therefore found for *E. marinus*, but not for *G. pulex*.

These findings are different from the available literature regarding the effects of pollutants on sperm in amphipod crustaceans. Studies have documented significant reductions in the sperm counts of amphipods exposed to endocrine disruptors (Gismondi et al., 2017; Trapp et al., 2014), cadmium (Trapp et al., 2014) and industrial pollution (Yang et al., 2008) though none of these studies considered sperm quality. These studies used pyriproxyfen and cyproterone acetate which have been shown to have deleterious impacts on the reproductive system of crustaceans at low doses (Ginjupalli and Baldwin, 2013) due to their role as juvenile hormone analogs. Similarly, cadmium is an extremely potent toxicant, with concentrations causing a significant reduction in sperm counts in the previous study of *Gammarus fossarum* (Trapp et al., 2014) spanning an environmentally relevant range of freshwater systems in Europe (Pan et al., 2010). Comparatively, the majority of studies recording a reduced sperm count in response to radiation exposure in biota used acute doses orders of magnitude higher than in the present study (Centola et al., 1994; Rowley et al., 1974). However, a number of studies have documented radiation effects on sperm numbers at lower doses. These studies assessed radiation-induced impacts on sperm in fish (Knowles, 1999) and birds (Møller et al., 2008, 2014) which are generally thought to be more radiosensitive than invertebrates (Hinton et al., 2007). In addition, these studies considered biota at Chernobyl (Møller et al., 2008, 2014) or longer-term laboratory exposures (Knowles, 1999), leading to accumulated doses orders of magnitude higher than the present study which may account for observed differences between studies.

One possible factor for the observed effects on sperm quality but not quantity in *E. marinus* may be the nature of the specific pollutant and the technique used to assess sperm quality. The LIVE/DEAD viability assay assesses sperm plasma membrane integrity, which is known to be sensitive to reactive oxygen species (ROS) that are generated indirectly following radiation exposure. Sperm plasma membranes are rich in polyunsaturated fatty acids (Cerolini et al., 2000; Duru et al., 2000) which are susceptible to lipid peroxidation (oxidative degradation of lipids), leading to loss of membrane integrity. However, modelling studies of the radiation-induced oxidative impacts of 10 mGy/d dose rates (the maximum used in this study) have indicated that such dose levels would not significantly impact cellular concentrations of ROS (Smith et al., 2012). These modelling studies have been supported by experimental data obtained for the freshwater crustacean *Daphnia magna* (Gomes et al., 2018), wherein dose rates significantly higher than the present study of 26.4 mGy/d and 256.8 mGy/d were needed to elicit ROS and lipid peroxidation respectively. Taking this and the lack of a significant effect in the closely related *G. pulex* at similar dose rates, further mechanistic studies are therefore required to elucidate the drivers behind the reduced sperm quality observed in the present study.

3.3. DNA damage

Owing to clear effects on sperm quality over two repeated exposures and greater survival of the marine compared to the freshwater

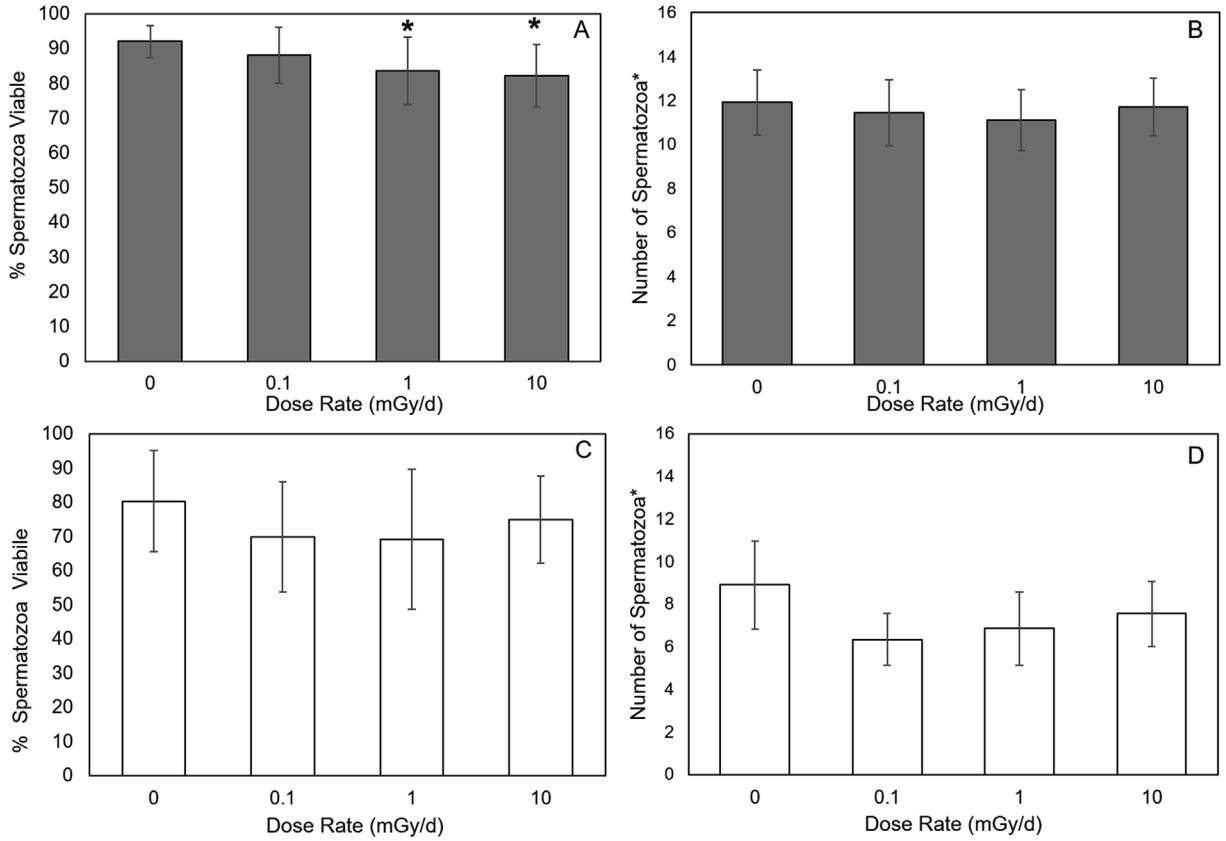

Fig. 1. Mean fourth root transformed sperm counts (A) and viability (B) in *Echinogammarus marinus* (N = 148). Mean fourth root transformed sperm counts (C) and viability (D) in *Gammarus pulex* (N = 61) following 14 day exposure to phosphorus-32 at dose rates of 0, 0.1, 1 and 10 mGy/d. * Indicates significant difference from the control (Pairwise comparisons, Bonferroni correction, $p < 0.05$). Error bars are ± 2 Standard Errors.

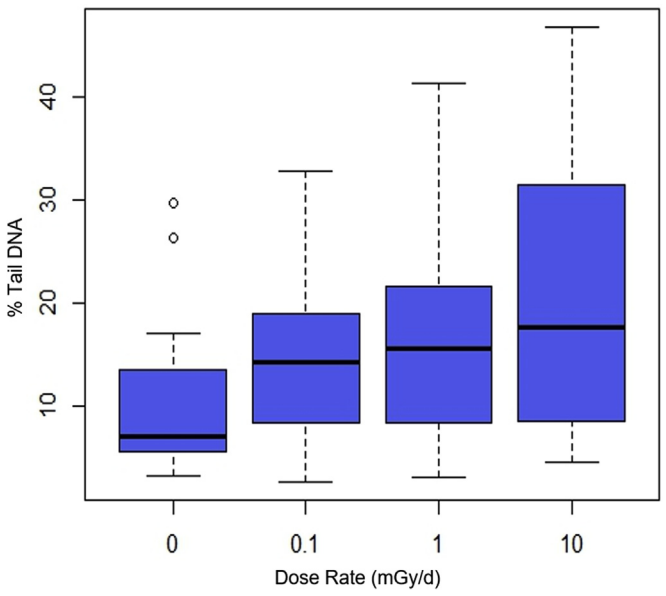

Fig. 2. Boxplot of the relationship between dose rate and DNA damage (% Tail DNA) in *Echinogammarus marinus* spermatozoa. The median, first and third quartile and outliers (O) are shown.

speciesunder experimental conditions, follow-on experiments on DNA damage and breeding were conducted on *E. marinus* only. Radiation dose rate had a significant (ANOVA, $F_{1, 66} = 4.987$, $p < 0.05$) effect on DNA damage in *E. marinus* spermatozoa. DNA damage increased with increasing dose rate, with the lowest % tail DNA recorded in the control organisms (10.30 ± 7.51%) and the greatest in the 10 mGy/d treatment (20.69 ± 12.74%, see Fig. 2). Subsequent post-hoc pairwise comparisons found significant differences in DNA damage between control and 10 mGy/d treatments only (Pairwise comparisons, Bonferroni correction, $p < 0.05$). A weak but significant positive relationship was recorded between log transformed DNA damage and dose rate ($R^2 = 0.071$, $F_{1, 66} = 4.978$, $p < 0.05$).

Though radiation exposure caused an increase in sperm DNA damage in *E. marinus*, this was only significant relative to controls in the 10 mGy/d treated group. This may be due to elevated DNA repair at higher dose rates. It has been hypothesised that a threshold exists for radiation-induced activation of DNA repair, which has been demonstrated in fish (Grygoryev et al., 2013). Similarly, induction of DNA repair following radiation exposure has been observed in copepods exposed to acute high doses of gamma radiation (Won and Lee, 2014). In spermatozoa however, mammalian studies have demonstrated reduced DNA repair capacity (Aitken and De Iuliis, 2007). During the late phase of spermatogenesis, DNA repair is generally down-regulated leading to reduced repair capabilities in sperm. In amphipods, Lacaze et al. (2011b), found no reduction in DNA damage in *Gammarus fossarum* sperm 4 days post exposure to genotoxicants, suggesting limited DNA repair capacity. Conversely, Lewis & Galloway (Lewis and Galloway, 2009) demonstrated recovery of sperm cells in *Mytilus edulis* 72 h post-exposure to MMS, though recovery was lower than in somatic cell types. No studies have assessed sperm DNA damage and repair in aquatic invertebrates exposed to ionising radiation. This study provides novel data regarding the impacts of environmentally relevant doses of radiation on DNA integrity in aquatic invertebrate sperm. Given the clustered, complex and unique signature of DNA damage induced by IR (see Lomax et al. (2013), for review), further studies of DNA repair in aquatic invertebrate germ cells are necessary to contextualise the

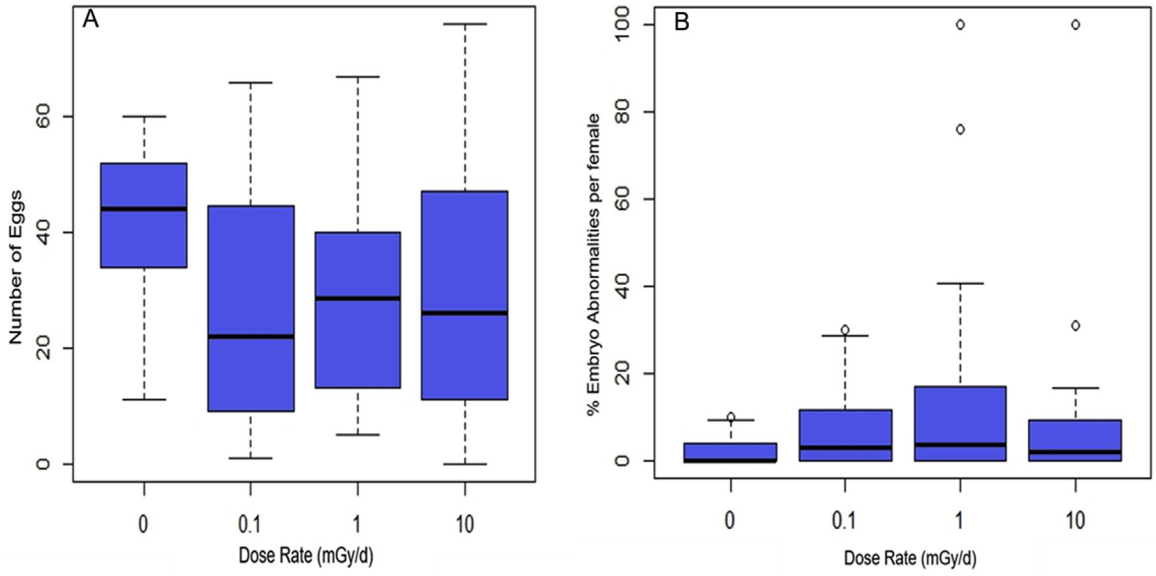

Fig. 3. Boxplot of the relationship between A) male radiation exposure and resultant number of eggs following reproduction with an unexposed female and B) Percentage of abnormal embryos (total number of malformed embryos/total number of embryos per female (Camus & Olsen, 2008) in female *E. marinus* breeding with males exposed to radiation. The median, first and third quartile and outliers (O) are shown.

findings of the present study.

3.4. Knock-on impacts on female reproduction

Radiation exposure had no significant effect on the time taken to reproduce following pairing with an unexposed sexually mature female (Kruskal Wallis H-test, $\chi^2 = 7.661$, df = 3, $p > 0.05$). Female *E. marinus* produced on average 32.37 ± 19.95 eggs across all treatments. Male exposure to radiation was found to have a significant effect on resultant fecundity in *E. marinus,* owing to significantly smaller brood sizes at all dose rates relative to controls (see Fig. 3A, ANCOVA, $F_{4, 60} = 4.296$, $p < 0.05$). Females breeding with males from the control group produced the greatest number of eggs (41.56 ± 3.39), with the fewest produced by females breeding with 0.1 mGy/d treated animals (28.23 ± 5.86). Male radiation exposure had no significant effect on embryo diameters following reproduction (Kruskal Wallis H-test, $\chi^2 = 2.130$, df = 3, $p > 0.05$).

A significant increase in the frequency of embryo abnormalities per female was recorded in females breeding with males exposed to 1 and 10 mGy/d relative to controls (2 × 4 Contingency Table, $\chi^2 = 13.085$, df = 1, $p < 0.05$ and $\chi^2 = 11.966$, df = 1, $p < 0.05$ respectively). This was largely due to the presence of 'dead' broods where 100% of the brood is abnormal (see Fig. 3B (Sundelin and Eriksson, 1998),). To assess the contribution of dead broods to the observed relationship, the data was also analysed following exclusion of samples exhibiting 100% embryo abnormalities. In this case, a significant increase in abnormalities relative to controls was observed only between 0 and 1 mGy/d, with no significant differences between any other treatments.

A significant positive relationship between sperm viability and weight-normalised egg numbers was recorded (see Fig. 4A, Spearmans rho = .280, $p < 0.05$), suggesting effects observed on sperm quality may have been the driver for reduced fecundity following reproduction. Sperm quality has been demonstrated to be a key determinant of fertilization success in a range of aquatic organisms including fish (Casselman et al., 2006), urchins (Au et al., 2001) and polychaete worms (Lewis et al., 2008) In amphipod crustaceans, the potential for reductions in sperm count to have significant effects on resultant fecundity has been highlighted using a modelling approach in Ford et al. (2012).

However, the magnitude of the effect on fecundity was greater than those observed for sperm quality, suggesting that another factor may have contributed to the effects on breeding. For example, significant reductions in brood sizes in females breeding with males exposed to 0.1 mGy/d were observed, wherein only subtle non-significant effects (4% reduction in sperm quality) were observed. In *E. marinus*, brood sizes typically range from 11 to 40 eggs (Cheng, 1942) and vary significantly even within females of the same size class. The fecundity results were characterised by high inter-individual variability, especially in radiation exposed groups (see Fig. 3A), which may have obscured any dose-response relationships. Given the limited sample size and high inter-individual variability, further research of the impacts of male radiation exposure on subsequent fecundity is necessary.

A significant negative relationship between the % of individuals developing normally and the percentage of DNA damage was recorded (Spearmans rho = −.310, $p < 0.05$, see Fig. 4B). This suggests that paternal IR exposure and resultant sperm DNA damage may be the mechanism for observed effects on post-fertilisation embryo abnormalities. However, no significant relationship was recorded following removal of the individuals exhibiting 100% embryo abnormalities, though this approached significance (Spearmans rho = −0.220, $p = 0.09$). Furthermore, given the absence of a clear dose-response relationship and the lack of experimental replication for this component of the research, robust conclusions cannot be drawn.

Significant relationships between sperm DNA damage and the frequency of abnormalities in resultant embryos have been documented for aquatic invertebrates (Lewis and Galloway, 2009), fish (Devaux et al., 2011) and bulls (Fatehi et al., 2006) These studies used model genotoxicants including methyl methanesulfonate (MMS), benzo(a) pyrene and high, acute doses of x-rays and gamma radiation respectively (doses up to 300 Gy). By contrast, this study is the first to the authors' knowledge to suggest such effects following exposure to environmentally relevant doses of ionising radiation, though experimental replication is required to confirm these findings. This phenomenon is thought to be due to persistence of germ cell mutations through embryonic cell generations, leading to transmissions of paternal genetic damage (Luke et al., 1997). Two principal mechanisms for the transmission of genetic damage have been proposed: induction of germ-line genomic instability or suppression of germ cell apoptosis (see [80]for review). A recent transgenerational study of *Daphnia magna* exposed to chronic gamma radiation found transmission of epigenetic changes to

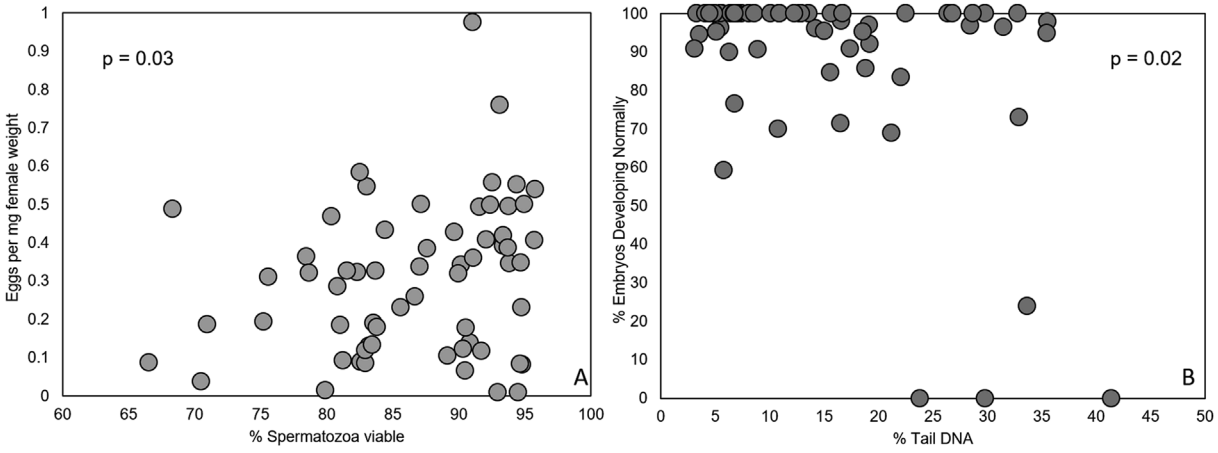

Fig. 4. A) Relationship (Spearmans rho = .280, $p < 0.05$) between *Echinogammarus marinus* sperm viability and resultant weight-normalised fecundity following breeding and B) Relationship (Spearmans rho = −0.310, $p < 0.05$) between DNA damage in the sperm of *Echinogammarus marinus* and development in resultant embryos following reproduction.

unexposed generations, which was suggested to be through the germline (Trijau et al., 2018). Epigenetic changes as outlined in Trijau et al. (2018), have been suggested to be a potential cause of genomic instability in *D. magna*, which may explain the transmission of effects observed in the present study. Recent studies in humans and animal models have suggested a role of epigenetic modifications to sperm DNA in determining effects on the resultant embryos (see Stuppia et al. (2015), for review). Taking this into account, further mechanistic study of the link between sperm DNA damage and resultant development is necessary using an epigenetic approach.

One key limitation of the present work was that individual exposures were conducted for each of the three experimental components as opposed to performing direct fertilizations with sperm within which DNA damage and viability had been assessed. Coupled with natural inter-individual variability in the measured parameters such as sperm counts (e.g. (Yang et al., 2008)) and fecundity (Cheng, 1942), this may explain the weak relationships observed between sperm parameters and resultant fecundity and embryo parameters. In amphipod crustaceans, fertilization occurs semi-externally meaning it is not possible to collect ejaculates (Lemaître et al., 2009). Samples of sperm can be collected only by dissection (Gismondi et al., 2017), necessitating individual exposures for each component of the present work.

4. Conclusions

Significant effects on sperm quality and resultant fecundity in *E. marinus* were recorded at doses of 1 mGy/d. This value is below the commonly used derived consideration reference level (DCRL) in the emerging system for environmental radioprotection proposed by the ICRP of 10–100 mGy/d (ICRP, 2008), wherein significant effects on crustacean populations are anticipated. This suggests that crustaceans may be more sensitive to radiation than previously thought, with implications for environmental radioprotection. However, the goal of the majority of environmental protection benchmarks is the protection of populations of organisms. Though the effects recorded in the present work could potentially have deleterious impacts at higher ecological levels, given the lack of a clear dose-response relationship and different responses between two closely related species, further work is necessary to determine the likelihood of such effects occurring in the natural environment. In addition, the response of the invertebrate population to these subtle reproductive effects is likely to be masked by density-dependent factors: each *E. marinus* female produces between 11 and 40 offspring in her lifetime (Cheng, 1942) of which (for a stable population) only a limited number will survive to produce viable offspring.

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